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(54) PREPARING PROTEIN-CONTAINING MATERIAL

(71) We, DAI-NIPPON SUGAR MANUFACTURING COMPANY, LIMITED, a Japanese Company, of No. 1–5–1, Marunouchi, Chiyoda-ku, Tokyo, Japan, do hereby declare

the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a method of preparing a protein-containing material which may be used to produce a substantially tasteless and odourless protein-containing beverage or a raw material therefor from proteinous cells such as of microorganisms or cereals.

Various protein-containing beverages are known, e.g., soybean juice. However, these beverages have not yet become popular because of their inherent unpleasant strong odour, and their astringent and bitter taste.

The use of a cell membrane lytic enzyme has been proposed for eluting intracellular substances, but this conventional method requires a prolonged time for the lysis of the cell membrane, and at the same time the protein is partially decomposed into dipeptides or even into amino acids, thus generating unpleasant taste and odour.

According to the present invention, microorganisms or cereals, such as beans (for example, peanuts and soybean) or defatted cakes thereof, and grains such as corn, wheat and oats (or protein-rich portions thereof) are extracted with at least one of water at a temperature of from 60 to 150°C, an acid, an alkali or an organic polar solvent and the cell membrane in the resulting insoluble residue is ruptured using a cell rupturing machine such as a sand grinder in the presence of at least one cell membrane lytic enzyme and/or proteolytic enzyme. Thus, the cell membrane may be mechanically ruptured with ease, and the activity of the cell membrane lytic enzyme or proteolytic enzyme and the like may be greatly increased whereby solubilization and dispersion of the cell membrane together with interacellular substances may be readily achieved in a short period of time. In addition, a starch hydrolyzing enzyme and/or a lipolytic enzyme may optionally be used together with the above enzymes. When the mechanical rupturing treatment is effected in combination with the enzymatic lysis using these enzymes, a slight increase of viscosity and a extremely increased dispersibility of the resulting liquid can result and the cell membrane may be dispersed in the form of fine particles whereby a homogeneous protein-containing beverage or a raw material therefor which is substantially free from unpleasant taste and odour can be obtained.

Raw materials which are used in the present invention are microorganisms or cereals, such as beans or defatted cakes thereof, maize and grain, e.g., wheat, oat and barley. Microorganisms which may be employed as a raw material in the present invention include yeasts such as *Candida utilis* (generally called Torula yeast), *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Saccharomyces fragilis*, *Candida tropicalis*, *Candida lipolitica* and *Rhodotorula glutinis*, bacteria such as *Bacillus subtilis* and *Corynebacterium* sp., blue-green algae such as Arthorospira and Spirulina, and green-algae such as *Chlorella vulgaris* and *Scenedesmus* sp. When it is desirable to obtain products having a low content of starch from those materials having a high starch content, they may be used after most of the starch has been removed therefrom by, for example, a direct starch-separating method as is commonly employed in the art, such as alkali treatment or sulphurous acid treatment. Cereals are preferably used in the form of a powder having a particle size less than 60 mesh (Taylor Standard).

Suitable concentrations of the raw materials in the extraction with hot water, an acid, an alkali or a polar solvent are generally less than 25%, preferably from 5 to 20%, by weight based on the dry solid content.

Extraction with hot water, if employed, is carried out at a temperature of from 60

to 150°C, if necessary, under pressure, generally for a period of less than about 120 minutes.

Acid extraction, if employed, may be carried out using inorganic acids, for example hydrochloric acid, sulphuric acid, phosphoric acid or carbonic acid or organic acids, for example citric acid, lactic acid or acetic acid at a pH of from 5.5 to 1, preferably 5.5 to 3.0, at a temperature of 98 to 30°C for a period more than 5 minutes, preferably from 10 minutes to 5 hours.

Alkalis which may be used in the present invention includes ammonia and hydroxides, carbonates, lactates, citrates, succinates, and acetates of the cations of sodium, potassium, ammonium, magnesium and calcium. The extraction with these alkalis if employed, may be carried out at a pH of from 6.5 to 13, preferably 7.5 to 12.5, at a temperature of from 0 to 55°, preferably 3 to 40°C, for more than 5 minutes, preferably from 10 minutes to 5 hours. If necessary, the protein which has been eluted into the extraction liquid can be recovered by treating the liquid with trichloroacetic acid or by adjusting the pH of the liquid to an isoelectric point of the protein to precipitate the eluted protein whereby the loss of the protein during the extraction is minimized and the overall yield of protein-containing beverages may be increased.

The extraction with organic polar solvents, other than the organic acids mentioned above, if employed, may be conducted using, for example, alcohols such as methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol, or tert-butanol, at an alcohol concentration of greater than 30% by weight, particularly 75 to 90%, at a temperature generally below 100°C, preferably 30 to 70°C, for from 5 to 120 minutes. Extraction with a polar solvent brings about good results for all materials contemplated in the present invention in that the resulting products are substantially free from upleasant taste and odour. When it is desirable to defat raw materials, particularly microorganisms, the defatting procedure can effectively be carried out using organic solvents such as n-hexane or ethyl ether which can usually be employed either before, after or simultaneously with an above polar solvent treatment without any adverse effect.

The cell membrane of the materials thus left as residue is then ruptured and at the same time solubilized and dispersed by a rupturing machine such as a sand grinder, an impact-cell-mill or a three-roll-mill in the presence of at least one cell membrane lytic enzyme and/or a proteolytic enzyme.

The cell membrane lytic enzymes which may be used in the present invention can be those obtained from plants, animals and microorganisms. When these enzymes are used in a combination thereof, for example, a combination of Lysozyme with Cellulase-Onozuka, better results may be obtained.

As proteolytic enzymes which may be used in the present invention, proteinase, i.e., Papain, Bioplase (trade name, manufactured by Nagase Sangyo Co., Ltd., Japan), Panprosin (the optimum activity being in the vicinity of pH 2 to 6) and Bromelin, are suitable. A combined use of these cell membrane lytic enzymes with proteolytic enzymes increases the effect on solubilization and dispersion of the resulting products.

In addition, lipolytic enzymes, e.g., Lypase-My (trade name, manufactured by Meito Sangyo Co., Ltd., Japan) may optionally be employed with the above enzymes. Furthermore, when a starch-rich product is desired, a starch hydrolyzing enzyme such as Spitase-K (trade name for α-amylase, manufactured by Nagase Sangyo Co., Ltd., Japan) may be incorporated to liquefy the starch thereby increasing viscosity and ensuring dispersibility and good texture of the product. Incorporation of this starch hydrolyzing enzyme may be made simultaneously with the above cell membrane lytic enzymes, proteolytic enzymes or lipolytic enzymes, but it is most preferable to add a starch hydrolyzing enzyme in the final stages, i.e., after completion of the rupture treatment.

The total amount of enzymes to be used in the present invention is not critical and is generally from 0.1 to 19% by weight based on the solid content.

During the rupture treatment attention should be paid to the control of pH values and temperatures in view of optimum conditions of enzymes used. Suitable pH values are generally from 2 to 10, preferably 4.5 to 9, and the temperature is generally from 30° to 65°C, preferably 40° to 55°C. When a starch hydrolyzing enzyme is added to the liquid after completion of the rupture treatment as described above, it will be apparent that the temperature of the treated liquid may be increased as high as 80 to 95°C, which is the optimum tetmperature for this enzyme.

It is preferable to complete the rupture treatment in from 0.5 to 2 hours. If the rupture time is too long a slight bitter taste results but this may be removed by further adding acidic carboxypeptidase and the like produced by *Aspergillus* genus. Cell membrane rupturing machines which may suitably be used in the present invention include a sand grinder containing beads and the like, a sonicater and an impact-cell-mill. According to a combined use of a rupturing machine with enzymes, the membrane of cells can easily be ruptured and dispersed in a short period of time. The rupture end point may simply be determined by a change in taste, but since the object of the present in-

vention resides in solubilization and dispersion of proteinous cells, more accurate determination of the end point of the rupture may be made using a microscope or a BX meter or by observing sedimentation. When the product thus obtained is dried, saccharides such as dextrine, lactose, sucrose, glucose or the like, defatted milk, lactic acid or surface active agents may be incorporated into the product in order to obtain a dry product having good solubility in water and good dispersibility.

Moreover, the present invention also provides digestible raw materials or additives for skim milk substitutes, lactic acid beverages, Lactobacillus beverages, sake additives, cold cakes, refreshing drinks, fruit drinks and the like as well as nutritious protein-containing beverages *per se*.

The present invention will now be illustrated in greater detail by the following Examples.

EXAMPLE 1

500 Grams of dry Torula yeast was extracted with 4 portions (each of 2 litres) of 80% aqueous ethanol at 50°C for 30 minutes each time. Ethanol remaining in the Torula yeast was removed and 2500 ml of water was added to the yeast. 10 g of Cellulase-Onozuka P-1500 was added thereto while maintaining the temperature at 45°C. The resulting mixture was then placed in a sand grinder kept at a temperature of 45°C to carry out cell membrane lysis simultaneously with cell membrane rupture for a period of 20 minutes after which the mixture was allowed to stand for a period of 70 minutes. To the resulting product was added 100 g of sucrose and the mixture was then spray-dried to obtain 265 g of a light yellow product. The dried product displayed excellent solubility in water and did not have an unpleasant taste or odour.

EXAMPCE 2

To 300 grams of a defatted soybean powder was added 1 litre of water, and the mixture was thoroughly mixed in a kneader at 98°C for 30 minutes. To the resulting mixture was added 500 ml of water followed by 30 g of Cellulase-Onozuka P-1500, 0.2 g of Lypase-My and 0.1 g of Papain, and the mixture was allowed to stand for 90 minutes. The resulting mixture was then placed in a sand grinder maintained at 45°C and was subjected to cell rupture treatment for 30 minutes. The enzyme was inactivated by heating the mixture at 80°C for 10 minutes. After taking out the mixture from the sand grinder, the mixture was cooled to 50°C and 9 litres of ethanol was added thereto to mature precipitation. The precipitate thus formed was then filtered and 5 litres of ethanol was added to the filter cake followed by heating at 40°C for 60 minutes. After filtration and drying, 360 ml of water containing 3 g of Spitase-K, a starch hydrolyzing enzyme, was added to the mixture followed by stiring at 90°C for 20 minutes in a kneader to obtain a paste-like product. The product was diluted with water to twice the original volume. It was found that the product thus obtained had an extremely good solubility in water, good dispersibility and increased aromaticity with neither bitter taste nor soybean odour.

EXAMPLE 3

300 Grams of dry Torula yeast was extracted with 1.5 litres of hot water at 90°C for 30 minutes. The insoluble residue was further treated with 1 litre of hot water at 90°C for an additional 30 minutes. To the residue were added 0.2 litres of water, 15 g of Cellulase-Onozuka, 0.2 g of Lypase-My and 1 g of Takadiastase, and the resulting mixture was subjected to rupturing treatment using a sand grinder at a temperature of from 40 to 50°C for 20 minutes. The mixture thus treated was allowed to stand for 90 minutes followed by again subjecting to rupturing treatment for an additional 10 minutes. A 100 g portion of the resulting mixture was sterilized and 50 g of a 15% defatted milk solution, 30 g of a 30% sucrose solution and 5 g of a 20% yeast extract which had been separately sterilized were added thereto. The resulting mixture was inoculated with a lactic acid bacterium, and incubation was conducted for 5 hours at a temperature of 30°C. 10 ml of lactic acid (75%) and 10 g of sucrose were then added to prepare a lactice acid bacterium beverage. This beverage was found to have almost no yeast-odour and to be nearly white with good taste.

EXAMPLE 4

To 100 g of the protein-containing sterilized mixture obtained in Example 3 was added 15 g of fresh, cultured and compressed yeast of *Saccharomyces cerevisiae*, and cultivation was conducted for 3 hours at 28°C. After centrifuging, the supernatant liquid was taken out and sterilized by heating. 5 g of a defatted milk powder and 10 g of sucrose were added to the resulting liquid to prepare a milk substitute which was found to be delicious without any unpleasant taste.

EXAMPLE 5

1 Kilogram of fresh Torula yeast was extracted with 1 litre of ethanol for 60 minutes at room temperature with stirring. The mixture was filtered and the filter cake was further treated with 2 litres of ethanol for 60 minutes at 50 to 60°C with stirring. The residue was further extracted with 2 litres of *n*-hexane for 60 minutes at 50 to 60°C with stirring. After drying the resulting

residue, 700 g of water, 10 g of Meicelase, 0.3 g of Lysozyme and 2 g of Diastase were added thereto after which the mixture was subjected to rupturing treatment using a sand-grinder for 120 minutes at 45 to 50°C. To this was added sodium hydroxide solution to adjust the pH to 6.8. The liquid was then heated at 80°C for 10 minutes after which 2 g of sodium chloride and 200 g of sucrose were added, and the mixture was then spray-dried to obtain 160 g of a white yellow powder.

Rupturing was effected in the same manner as described above but using 0.2 litres of water in place of the ethanol and the *n*-hexane as used above, the mixture was heated at 80°C for 30 minutes and subjected to an enzyme treatment and rupturing treatment using a sand grinder simultaneously. The resulting mixture was adjusted to a pH of 6.8 and 2 g of sodium chloride and 200 g of sucrose were added thereto. The mixture was then spray-dried to obtain 106 g of a yellow-brown powder.

A 20% aqueous solution was prepared from these two samples as obtained above, respectively, and presented to an organoleptic test by panelists. The sample treated with ethanol was judged to exhibit no upleasant taste or odour, but the sample which had been heat-treated at 90°C for 30 minutes was judged by all panelists to have a bitter and astringent taste and a strong yeast odour.

WHAT WE CLAIM IS:—

1. A method of preparing a protein-containing material, which comprises extracting microorganisms or cereals with at least one of water at a temperature of from 60 to 150°C, an acid, an alkali and an organic polar solvent to obtain an insoluble residue, mechanically rupturing the membrane of cells contained in said residue in the presence of a cell membrane lytic enzyme and/or a proteolytic enzyme thereby releasing intracellular substances from said cells.

2. A method as claimed in Claim 1, wherein a cereal is employed which is selected from beans, defatted cakes thereof, maize, wheat, oat and barley.

3. The method as claimed in Claim 1 or 2, wherein said cereal is in the form of powder having a particle size of less than 60 mesh (Taylor).

4. A method as claimed in Claim 1, wherein a microorgonism is employed which is a yeast selected from *Candida utilis, Saccharomyces cerevisiae, Saccharomyces fragilis, Saccharomyces carlsbergensis, Candida tropicalis, Candida lipolitica* and *Rhodotorula glutinis*, a bacterium selected from *Bacillus subtilis* and *Corynebacterium* sp. or an algae selected from *Arthrospira, Spirulina, Chlorella vulgaris* and *Scenedesmus* sp.

5. A method as claimed in any preceding Claim, wherein said microorganism or cereal has a concentration less than 25% by weight based on the dry solid.

6. A method as claimed in Claim 5, wherein said concentration is from 5 to 20% by weight based on the dry solid.

7. A method as claimed in any preceding Claim, wherein extraction with hot water is employed at a temperature of from 60° to 150°C for a period of less than 120 minutes.

8. A method as claimed in any preceding Claim, wherein an extraction with an acid is employed, using at least one acid selected from hydrochloric acid, phosphoric acid, carbonic acid, acetic acid, lactic acid and citric acid, at a pH of from 5.5 to 1 at a temperature of from 98° to 30°C for a period of more than 5 minutes.

9. A method as claimed in Claim 8, wherein the extraction is effected at a pH of from 5.5 to 3.0 for a period of from 10 minutes to 5 hours.

10. A method as claimed in any preceding Claim, wherein extraction with an alkali is employed, using at least one of ammonia and hydroxides, carbonates, lactates, citrates, succinates and acetates of sodium, potassium, ammonium, magnesium and calcium cations, at a pH of from 6.5 to 13 at a temperature of from 0° to 55°C for a period of more than 5 minutes.

11. A method as claimed in Claim 10, wherein the extraction is effected at a pH of from 7.5 to 12.5 at a temperature of from 3° to 40°C for a period of from 10 minutes to 5 hours.

12. A method as claimed in any preceding Claim, wherein an extraction with a polar solvent is employed, using at least one of methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, iso-butanol and *tert*-butanol at an alcohol concentration of greater than 30% by weight at a temperature of less than 100°C for 5 to 120 minutes.

13. A method as claimed in Claim 12, wherein the extraction is effected at an alcohol concentration of from 75 to 90% at a temperature of 30° to 70°C.

14. A method as claimed in any preceding Claim, wherein there is employed a proteolytic enzyme, being proteinase.

15. A method as claimed in any of Claims 1—13 wherein there is employed a proteolytic enzyme as in Claim 14, being Papain, Bioplase, Panprosin or Bromelin.

16. A method as claimed in any preceding Claim, wherein the rupturing is effected in the presence of a lipolytic enzyme and/or a starch hydrolyzing enzyme in addition to the cell membrane lytic enzyme and/or the proteolytic enzyme.

17. A method as claimed in any of Claims 1 to 15, wherein a starch hydrolyzing enzyme is added to the ruptured cells after completion of the rupturing.

18. A method as claimed in any preceding Claim, wherein the total amount of enzymes present is from 0.1 to 19% by weight based on the solid content of said residue.

19. A method as claimed in any preceding Claim, wherein the rupturing is conducted at a pH of from 2 to 10 and at a temperature of from 30° to 65°C.

20. A method as claimed in Claim 19, wherein the rupturing is conducted at a pH of from 4.5 to 9 and at a temperature of from 40° to 55°C.

21. A method of preparing a protein-containing material, substantially as hereinbefore described with refrence to any one of the foregoing Examples 1 to 5.

22. A protein-containing material when prepared by means of a method according to any preceding Claim.

23. A protein-containing material, substantially as hereinbefore described with reference to any one of the foregoing Examples 1 to 5.

24. A digestible starting material for preparing a drink, beverage or cake, which includes a protein-containing material according to Claim 22 or 23.

25. A starting material as claimed in Claim 24 and which is in a dry state.

26. A protein-containing drink, beverage or cake which includes a protein-containing material according to Claim 22 or 23 or which is prepared from a starting material according to Claim 24 to 25.

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